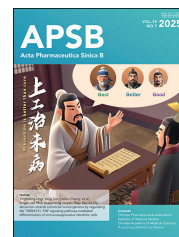




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## ORIGINAL ARTICLE

# Anacyphrethines A and B as potent analgesics: Multiple ion channel inhibitors with an unprecedented chemical architecture



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## KEY WORDS

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Natural products;  
Structure elucidation

**Abstract** Multi-target analgesics with minimal side effects and high efficacy are a key research focus in addressing the global pain crisis. Using a molecular networking approach, five pairs of potent analgesic alkaloid enantiomers were isolated from the roots of *Anacyclus pyrethrum* (*A. pyrethrum*). Their structures were elucidated by comprehensive spectroscopic data analysis, including LR-HSQMBC and <sup>1</sup>H–<sup>15</sup>N HMBC, quantum <sup>13</sup>C NMR DP4+ and ECD calculations, and single-crystal X-ray diffraction analysis. Anacyphrethines A (**1**) and B (**2**) are highly conjugated and polymethylated 6/6/6/5/7/5/5-fused octacyclic tetraazabicyclic alkaloids possessing an unprecedented 8,14,18,24-tetraaza-octacyclo [16.8.2.1<sup>1,23</sup>.0<sup>4,28</sup>.0<sup>5,17</sup>.0<sup>9,16</sup>.0<sup>11,15</sup>.0<sup>21,27</sup>] nonacosane motif. Their biosynthetic pathways are proposed involving key aldol, hydroamination, and Schiff base reactions. All isolates showed potent analgesic effects *in vivo*. Even at a lower dose of 0.2 mg/kg, (±)-**1** and (+)-**1** still exhibited more potent analgesic activities than morphine. Interestingly, the racemic mixture (±)-**1** showed stronger analgesic effect than either pure enantiomer alone at higher doses of 5 and 1 mg/kg; while, (±)-**1** showed significant analgesic activities comparable to (+)-**1** at lower doses of 0.2 and 0.04 mg/kg. (+)-**1** had stronger analgesic effect than (–)-**1** at five tested doses. Further tests on 44 analgesic-related targets demonstrated that (+)-**1** showed significant inhibitory effects against many ion channels such as TRPM8, Kv1.2, Kv1.3, and

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Ca<sub>v</sub>2.1 with IC<sub>50</sub> values of 1.10 ± 0.26, 4.20 ± 0.07, 2.20 ± 0.24, and 10.40 ± 0.69 μmol/L, respectively, while (–)-**1** primarily inhibited TRPC6, Kv1.2, and Kv1.3 ion channels with IC<sub>50</sub> values of 0.81 ± 0.05, 0.91 ± 0.04, and 1.50 ± 0.13 μmol/L, respectively, without affecting the opioid receptors, suggesting their non-opioid analgesic potentials. The molecular dockings provided structural guidance to develop potent non-opioid analgesics.

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## 1. Introduction

Pain is a defense mechanism of the body against injury or disease. However, persistent and severe pain has negative impacts on a patient's mental health, causing anxiety, sadness, and reduced immunity and, in the worst circumstances, may even be fatal or disabling<sup>1</sup>. Neuropathic pains and cancer-related pains, in particular, are still one of the most difficult to treat and severe types of pain in clinical practice<sup>2,3</sup>. Conventional analgesics, such as opioids and NSAIDs, are associated with various side effects, including drug resistance, addiction, and gastrointestinal bleeding<sup>4–6</sup>.

Currently, three major strategies have been reported to develop analgesic drugs, such as modifying opioids to reduce the risk of addiction and respiratory depression<sup>7–10</sup>, exploring new analgesic targets such as TRPM8<sup>11–13</sup>, TRPC6<sup>14–16</sup>, Kv1.2<sup>17,18</sup>, and Cav2.1<sup>19</sup> ion channels, implementing combinations of single-target analgesics<sup>20</sup>, and developing multi-target analgesics<sup>10</sup>. Very recently, the US FDA approved the first Nav1.8 ion channel inhibitor, suzetrigine (VX-548), as a novel non-opioid treatment for moderate to severe acute pains, marking the first novel-mechanism treatment in over two decades and advancing the development of non-addictive analgesics<sup>21</sup>. However, as a highly selective single-target antagonist, it may cause cardiovascular regulatory dysfunction as a potential side effect<sup>22</sup>. Among these strategies, the potential of multi-target drugs is demonstrated by their ability to weakly bind to multiple disease-related targets, thereby producing synergies that enhance therapeutic efficacy while reducing side effects and drug resistance<sup>23,24</sup>. Recently developed multi-target opioid analgesics, dezocine<sup>25</sup>, cebranopadol<sup>26</sup>, and BU08028<sup>27</sup> have made some headway in lowering opioid side effects like addiction and respiratory depression. However, their opioid resistance and side effects remain significant concerns in clinical use. Thus, the search for novel, potent analgesics with non-addictive properties and multi-target potential is urgently needed.

The roots of *Anacyclus pyrethrum* (*A. pyrethrum*), known as A-Na-Qi-Gen in Chinese, are used as a Traditional Chinese Medicine, especially in Xinjiang Uygur Autonomous Region, to treat headache, migraine headache, and cold-type toothache<sup>28</sup>. Preliminary phytochemical studies demonstrated the presence of piperidine alkaloids<sup>29</sup>. To search for novel alkaloids with potent analgesic activities, the roots of *A. pyrethrum* were re-investigated using a molecular networking strategy (Supporting Information Fig. S1), leading to the isolation of three pairs of new alkaloid enantiomers (Fig. 1), named anacyphrethines A–C (**1**–**3**), and two pairs of known alkaloid enantiomers pyracyclumines B (**4**) and A (**5**). Anacyphrethines A (**1**) and (**2**) are highly conjugated and polymethylated 6/6/6/6/5/7/5/5-fused octacyclic tetraazabicyclic alkaloids possessing an unprecedented 8,14,18,24-tetraaza-octacyclo[16.8.2.1<sup>1,2,3</sup>.0<sup>4,28</sup>.0<sup>5,17</sup>.0<sup>9,16</sup>.0<sup>11,15</sup>.0<sup>21,27</sup>] nonacosane motif. All the isolates showed potent analgesic activities. Among them,

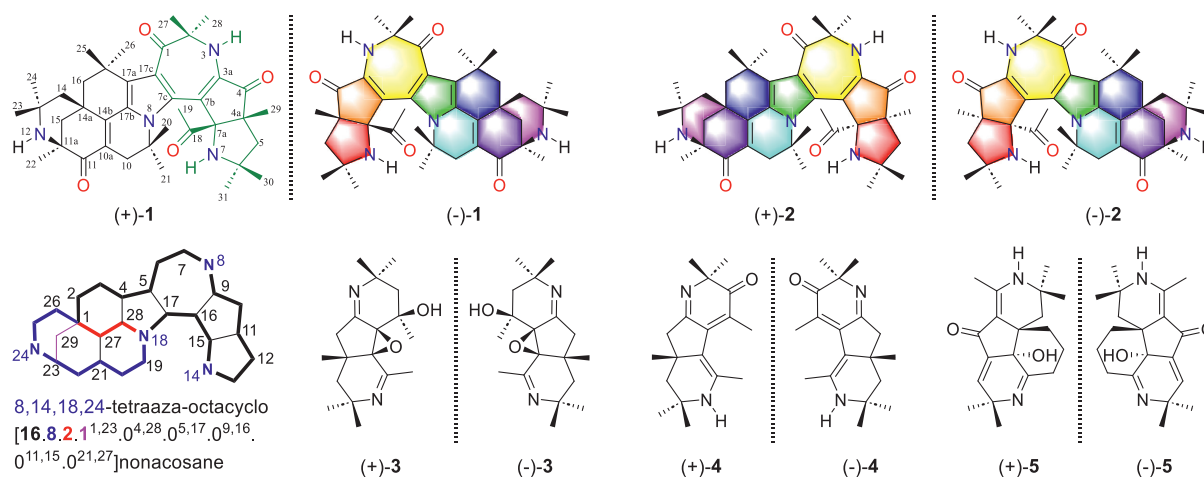
(±)-**1**, (+)-**5**, and (–)-**5** at a dose of 1.0 mg/kg and (±)-**1**, (+)-**1**, (±)-**3**, and (–)-**5** at a dose of 0.2 mg/kg exhibited more potent analgesic activities than morphine, the positive control. Further tests on 44 analgesic-related targets demonstrated that (+)-**1** showed significant inhibitory effects against many ion channels such as TRPM8, Kv1.2, Kv1.3, and Ca<sub>v</sub>2.1 with IC<sub>50</sub> values of 1.10 ± 0.26, 4.20 ± 0.07, 2.20 ± 0.24, and 10.40 ± 0.69 μmol/L, respectively, while (–)-**1** primarily inhibited TRPC6, Kv1.2, and Kv1.3 ion channels with IC<sub>50</sub> values of 0.81 ± 0.05, 0.91 ± 0.04, and 1.50 ± 0.13 μmol/L, respectively. Herein, isolation, structural elucidation, proposed biosynthetic pathways, and analgesic activities of **1**–**5**, along with the analgesic targets of (+)-**1** and (–)-**1** were presented.

## 2. Results and discussion

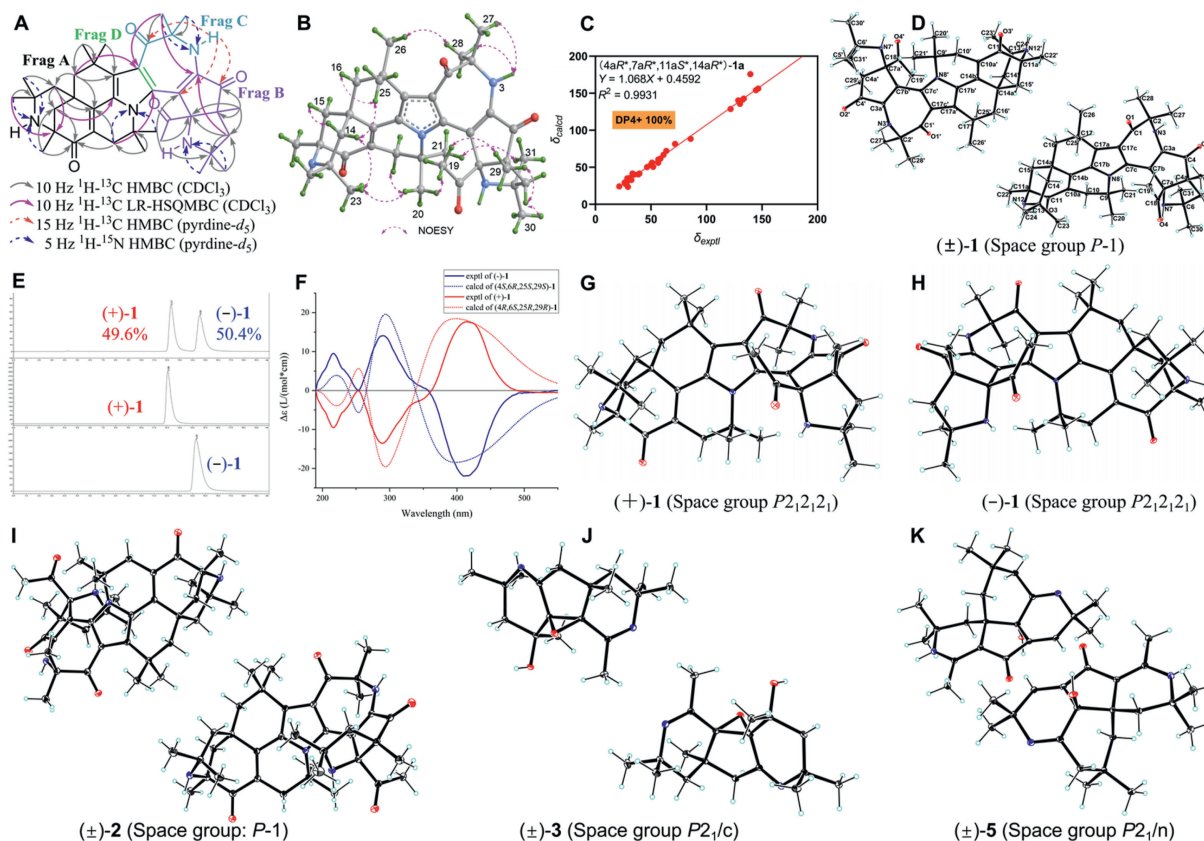
### 2.1. Structural elucidation of **1**–**5**

Anacyphrethine A (**1**) was isolated as a yellow amorphous powder. A molecular formula of C<sub>39</sub>H<sub>52</sub>O<sub>4</sub>N<sub>4</sub> was deduced from the (+)-HRESIMS ion at *m/z* 641.4051 [*M* + *H*]<sup>+</sup> (Calcd. for C<sub>39</sub>H<sub>53</sub>O<sub>4</sub>N<sub>4</sub>, 641.4066) and <sup>13</sup>C NMR data, indicating 16 degrees of unsaturation. The <sup>1</sup>H NMR data of **1** in chloroform-*d* (Supporting Information Table S1) displayed resonances for thirteen methyls and five methylenes, and <sup>13</sup>C NMR data (Table S1) exhibited 39 carbon resonances, assignable by DEPT135 and HSQC to be four carbonyls, eight olefinic carbons, nine quaternary carbons, five methylenes, and thirteen methyls. Four carbonyls and four double bonds account for 8 degrees of unsaturation, and the remaining 8 degrees of unsaturation suggested an octacyclic ring system in **1**.

Due to 21 non-protonated carbons and the lack of useful <sup>1</sup>H–<sup>1</sup>H COSY information, it is a great challenge to determine the structure of **1**. HMBC correlations (Fig. 2A) between H<sub>3</sub>-25/H<sub>3</sub>-26 and C-16/C-17a, between H<sub>2</sub>-14/H<sub>2</sub>-15/H<sub>2</sub>-16 and C-14a/C-14b, between H<sub>3</sub>-23/H<sub>3</sub>-24 and C-13/C-14, between H<sub>3</sub>-22 and C-11/C-11a/C-15, between H<sub>2</sub>-10 and C-10a/C-11/C-14b, and between H<sub>3</sub>-20/H<sub>3</sub>-21 and C-9/C-10 constructed a tetracyclic Frag A. The Frag B was defined by the HMBC correlations between H<sub>3</sub>-30/H<sub>3</sub>-31 and C-5/C-6, between H<sub>3</sub>-29 and C-4/C-4a/C-5/C-7a, between H<sub>2</sub>-5 and C-3a, between H<sub>3</sub>-19 and C-18/C-7a, and between H-7(N) and C-4/C-7b. The deshielding of C-2 (δ<sub>C</sub> 70.3) and the HMBC correlations between H<sub>3</sub>-27/H<sub>3</sub>-28 and C-1/C-2 demonstrated the presence of a nitrogen-containing dimethyl ketone motif (Frag C). However, there are no more useful HMBC correlations in chloroform-*d* to connect fragments A, B, and C due to the consecutive nitrogen atoms and non-protonated carbons. Based on the molecular formula and NMR data, **1** should have three nitrogenated protons (NH), however, only two NH (δ<sub>H</sub> 3.14,



**Figure 1** Chemical structures of **1–5** and the nomenclature of the unprecedented ring system.



**Figure 2** Structural elucidation of **1–3** and **5**. Key  $^1\text{H}$ - $^{13}\text{C}$  HMBC, LR-HSQMBC,  $^1\text{H}$ - $^{15}\text{N}$  HMBC (A), and ROESY (B) correlations, linear correlation plots between the experimental and calculated  $^{13}\text{C}$  NMR data for two isomers (C), ORTEP drawing of the crystal structures (D), chiral HPLC chromatogram (E), experimental and calculated ECD spectra of **1** and its enantiomer (F), and ORTEP drawings with 10% ellipsoid probability level of the crystal structures of (+)-**1** (G), (-)-**1** (H), ( $\pm$ )-**2** (I), ( $\pm$ )-**3** (J), and ( $\pm$ )-**5** (K).

7-NH; 4.17, 3-NH) were observed in the  $^1\text{H}$  NMR spectrum in chloroform- $d$ . To obtain more NH resonances and their HMBC information, NMR spectra of **1** were re-acquired in acetone- $d_6$ , DMSO- $d_6$ , and pyridine- $d_5$ , respectively. Fortunately, three NH resonances were observed at  $\delta_{\text{H}}$  5.56 (s, 3-NH), 3.44 (s, 7-NH), and 2.88 (s, 12-NH) in pyridine- $d_5$ . However, due to the electric quadrupole moment effects of the  $^{14}\text{N}$  core, both 3-NH and 12-NH

exhibited wide singlets, resulting in very weak HMBC correlations of NH.

To acquire more long-distance correlation signals, two pulse sequences hmbcglplndqf and hmbcetgpl3nd with different CNST parameters, were performed in the HMBC experiment. Fortunately, the key HMBC correlations of 3-NH to C-1/C-4/C-7b were observed under the hmbcglplndqf pulse sequence with the constants of CNST

[2] = 145 and CNST [13] = 15. Thus, the connection of fragments B to C through N-3 was established. The presence of four nitrogen atoms in the main skeleton of **1** made it difficult to connecting fragments A, B and C using HMBC. Thus,  $^{15}\text{N}$  NMR,  $^1\text{H}$ – $^{15}\text{N}$  HSQC, and  $^1\text{H}$ – $^{15}\text{N}$  HMBC spectra of **1** were acquired in pyridine- $d_5$ .  $^1\text{H}$ – $^{15}\text{N}$  HMBC correlations (Fig. 2A) of  $\text{H}_3$ -22/ $\text{H}_3$ -23/ $\text{H}_3$ -24 to 12-N constructed the piperidine ring of the fragment A.

To further construct the structure of **1**, LR-HSQMBC experiment (Fig. 2A) was used to get  $^4J$  and  $^5J$  long-distances correlations. LR-HSQMBC correlations of  $\text{H}_2$ -15 to C-17b and  $\text{H}_2$ -10/ $\text{H}_2$ -16/ $\text{H}_3$ -27 to C-17c established the connection of fragments A, B, C and D. Thus, the planar structure of **1** was defined as a highly conjugated and polymethylated 6/6/6/6/5/7/5/5-fused octacyclic tetraazabicyclic alkaloid bearing an unprecedented 8, 14, 18, 24-tetraaza-octacyclo[21.3.2 $^{1,4}$ .1.0 $^{2,21}$ .0 $^{3,18}$ .0 $^{5,17}$ .0 $^{9,16}$ .0 $^{11,15}$ ]nonacosane motif, its naming is followed the nomenclature of the ring system of IUPAC<sup>30</sup>.

The relative configuration of **1** was established by the NOESY data analysis in pyridine- $d_5$  (Fig. 2B) and DP4+ probability analysis<sup>31–34</sup>.  $\text{H}_3$ -29 ( $\delta_{\text{H}}$  1.16) was randomly assigned to be  $\beta$ -oriented. The NOESY correlations of  $\text{H}_3$ -29 to  $\text{H}_3$ -19 ( $\delta_{\text{H}}$  1.884),  $\text{H}_3$ -19 to  $\text{H}_3$ -20 ( $\delta_{\text{H}}$  1.47),  $\text{H}_3$ -20 to  $\text{H}_3$ -23 ( $\delta_{\text{H}}$  1.24), and  $\text{H}_3$ -23 to H-14a ( $\delta_{\text{H}}$  2.26) established the  $\beta$ -orientations of 7a-acetyl group and the *N*-ethyl bridge of the 2-aza-bicyclo[3.3.1]nonane motif. To further define the relative configurations of **1**, the NMR data of two possible isomers **1a** and **1b** were calculated using the gauge-independent atomic orbital (GIAO) method at the mPW1PW91/6-311G(d,p) level with the Gaussian 09 software<sup>35,36</sup>. Results (Fig. 2C) showed that (4aR\*, 7aR\*, 11aS\*, 14aR\*)-**1a** had better coefficient of determination ( $R^2 = 0.9931$ ) of the linear correlation between the experimental and calculated  $^{13}\text{C}$  NMR data than (4aS\*, 7aS\*, 11aS\*, 14aR\*)-**1b** ( $R^2 = 0.9899$ ). Further DP4+ probability calculation (Supporting Information Table S2) defined the relative configuration of **1** as 4aR\*, 7aR\*, 11aS\*, 14aR\* with a high probability of 100%. Finally, the structure of **1** was confirmed by single-crystal X-ray diffraction analysis, revealing its racemic nature by the symmetric space group (*P*-1) (Fig. 2D).

Two optically pure enantiomers (+)-**1** and (–)-**1** in a ratio of 1:1 were successfully obtained by chiral HPLC separation (Fig. 2E). To establish their absolute configurations, the ECD spectra of (+)-**1** and (–)-**1** were calculated at the LC-wPBE/6-311G(d,p) level with the Gaussian 09 software<sup>32,33,37,38</sup>. The experimental ECD spectrum of (+)-**1** fits well with the calculated spectrum of (4aR, 7aR, 11aS, 14aR)-**1** (Fig. 2F), while, the experimental ECD spectrum of (–)-**1** matches well with the calculated spectrum of (4aS, 7aS, 11aR, 14aS)-**1**. Therefore, the absolute configurations of (+)-**1** and (–)-**1** were defined as 4aR, 7aR, 11aS, 14aR and 4aS, 7aS, 11aR, 14aS, respectively. Finally, the absolute configurations of (+)-**1** and (–)-**1** were confirmed by the single-crystal diffraction with a Flack parameter of –0.01(5) (Figs. 2G) and 0.01(4) (Fig. 2H), respectively.

In the same way, anacypirethines B (**2**) and C (**3**) were determined to be a new 4a,7a-epimer of **1** and a new heptamethylated 6/5/6 fused tricyclic diazabicyclic alkaloid, respectively, based on comprehensive spectroscopic data analysis (Supporting Information Figs. S2–S6 and Supporting Information Tables S3–S5). Known compounds **4** and **5** were identified to be pyracyclumines B (**4**) and A (**5**), respectively, by spectroscopic data analysis (Supporting Information Tables S6 and S7) and by comparing their spectroscopic data with those reported in the literature<sup>29</sup>. The structures of **2**, **3** and **5** were confirmed by single-crystal X-ray diffraction analysis (Fig. 2I–K), revealing racemates by their centrosymmetric space groups. Compounds **2**–**5** were

successfully resolved by chiral HPLC (Fig. S4A and Supporting Information Figs. S6–S8), and the absolute configurations of the enantiomers of **2** and **3** were assigned by the ECD calculations (Fig. S4B and Fig. S6). The detailed structural elucidation of **2**–**5** was described in the Supporting Information

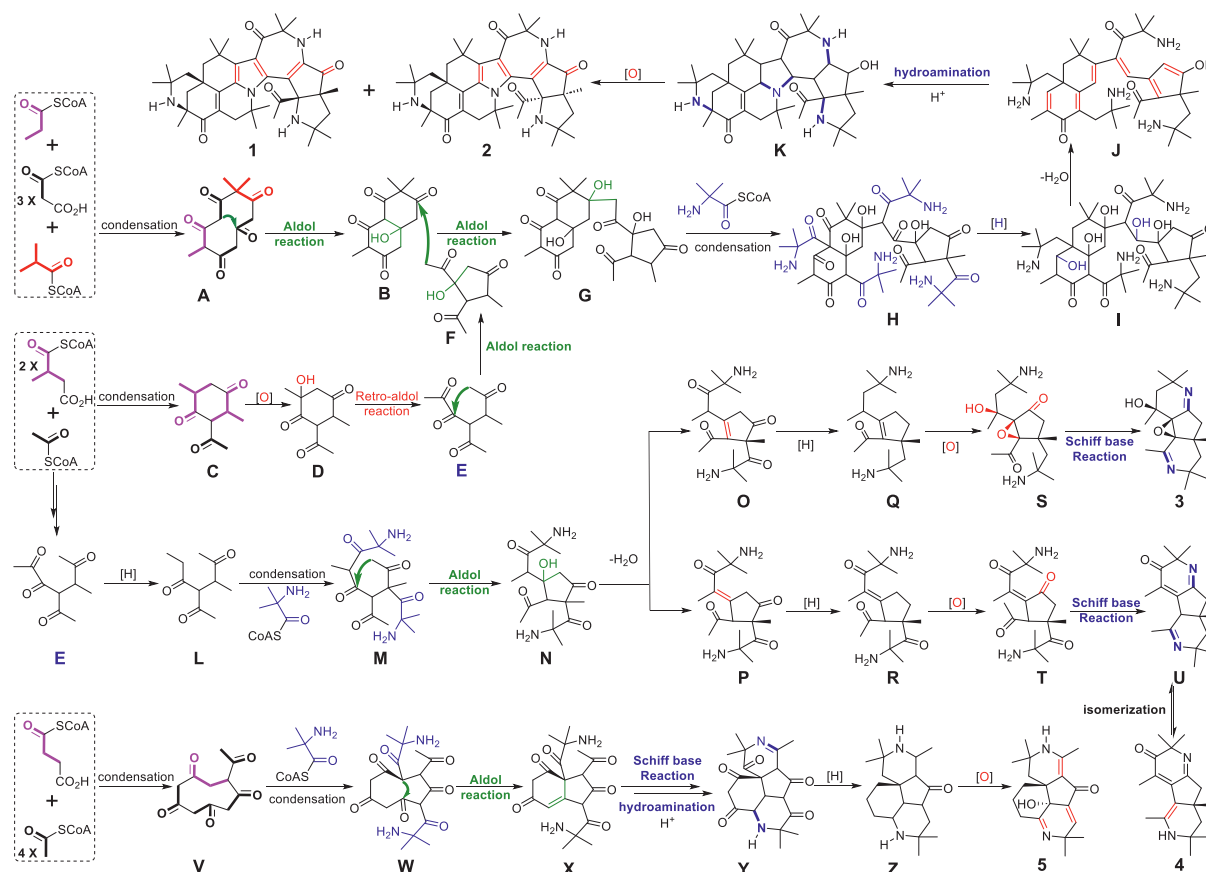
## 2.2. Proposed biosynthetic pathways for **1**–**5**

Anacypirethines A (**1**) and B (**2**) are two epimers of highly conjugated and polymethylated 6/6/6/6/5/7/5/5-fused octacyclic tetraazabicyclic alkaloids possessing an unprecedented 8, 14, 18, 24-tetraaza-octacyclo[16.8.2.1 $^{1,23}$ .0 $^{4,28}$ .0 $^{5,17}$ .0 $^{9,16}$ .0 $^{11,15}$ .0 $^{21,27}$ ]non-acosane motif. Anacypirethine C (**3**) and pyracyclumine B (**4**) shared a heptamethylated 6/5/6-fused tricyclic diazabicyclic skeleton, while pyracyclumine A (**5**) possessed a pentamethylated 6/6/5/6-fused tetracyclic diazabicyclic alkaloid core. The biosynthetic pathways for pyracyclumines A (**5**) and B (**4**) were previously proposed to be derived from piper alkaloids *via* a very complex procedure from a purely chemical transformation perspective<sup>29</sup>. However, structurally, alkaloids **1**–**5** are *N*-containing polyketides sharing a 1,1-dimethyl ethylamine unit, thus, 2-aminoisobutyryl-CoA is proposed to be their key building block in a biosynthetic view<sup>39</sup>. The biosynthetic pathways for **1**–**5** (Scheme 1) are proposed to initiate with a crucial condensation reaction between polyketides and 2-aminoisobutyryl-CoA.

The condensation of one propionyl-CoA, three malonyl-CoA, and one isobutyryl-CoA forms the ten-membered polyketide **A**, and the following intramolecular aldol condensation reaction yields the bicyclic polyketide **B**. Similarly, the six-membered polyketide **C** is produced by the condensation of two 2-methylsuccinyl-CoA and one acetyl-CoA. The five-membered polyketide **F** is derived from **C** through a series of transformations, including oxidation, retro-aldol cleavage, and aldol condensation. The aldol reaction of the methyl in **B** to the carbonyl in **F** forms the dimeric polyketide **G**, which reacts with four molecules of 2-aminoisobutyryl-CoA to generate the pivotal nitrogen-containing polyketide **H**. The highly conjugated polyketide **J** is derived from **H** by the reduction and dehydration reactions. Under the catalysis of acid enzymes, the intramolecular hydroamination reaction of the amine and the double bond in **J** generates the 6/6/6/6/5/7/5/5-fused octacyclic alkaloid **K**. The final oxidation of **K** by dehydrogenases affords anacypirethines A (**1**) and B (**2**).

The reduced product **L** derived from the linear polyketide **E** reacts with two molecules of 2-aminoisobutyryl-CoA to generate the diazabicyclic polyketide **M**, in which the intramolecular aldol reaction forms the five-membered nitrogen-containing polyketide **N**. The dehydration of the hydroxyl in **N** yields two regioisomeric alkenes **O** and **P**, which undergo reduction and oxidation in sequence to generate intermediates **S** and **T**, respectively. The intramolecular Schiff base reaction of the amino and the ketone carbonyl groups in **S** and **T** produces the imine group in the 6/5/6-fused tricyclic diazabicyclic alkaloid **3** and **U**, respectively. Pyracyclumine B (**4**) is derived from the double-bond isomerization of **U**.

Similarly, a succinyl-CoA and four molecules of malonyl-CoA are condensed to the nine-membered polyketide **V** (Scheme 1), which then undergoes condensation with two molecules of 2-aminoisobutyryl-CoA to generate the nitrogen-containing polyketide **W**. The intramolecular aldol reaction in **W** forms the key bicyclic polyketide **X**. The intramolecular Schiff base reaction of the amino and the ketone carbonyl groups and the hydroamination of the amino to the double bond produces the tetracyclic alkaloid **Y**. The following reduction, oxidation, and dehydration produces



Scheme 1 Proposed biosynthetic pathways for 1–5.

pyracyclumine A (**5**). All the isolates **1–5** are racemic mixtures, suggesting that some transformations such as the aldol condensation reaction are non-enzymatic.

The proposed biosynthetic pathways for **3–5** further support the rationality of the biosynthetic pathways for **1** and **2**. More importantly, these proposed biosynthetic pathways would provide valuable insights into the total synthesis and biosynthesis of **1–5**.

### 2.3. Analgesic activity evaluation of 1–5

Since the roots of *A. pyrethrum* are used as a Traditional Chinese Medicine to treat headache, migraine headache, and cold-type toothache, all the isolates **1–5** were evaluated for their analgesic activities in an acetic acid-induced writhing test in mice<sup>33,40</sup>. Results demonstrated that all the isolates showed significant analgesic activity at a dose of 5.0 mg/kg compared to the vehicle group (Fig. 3).

Among them, ( $\pm$ )-**1**, (+)-**5**, and (–)-**5** exhibited more potent analgesic activities than the positive control, morphine, at a dose of 1.0 mg/kg, with percentage inhibitions of  $83.7 \pm 1.7\%$ ,  $78.6 \pm 1.5\%$ , and  $74.9 \pm 1.9\%$  (Fig. 3), respectively. Meanwhile, ( $\pm$ )-**1**, (+)-**1**, ( $\pm$ )-**3**, and (–)-**5** exhibited more potent analgesic activities than morphine at a lower dose of 0.2 mg/kg, with percentage inhibitions of  $57.3 \pm 1.7\%$ ,  $61.5 \pm 2.4\%$ ,  $74.9 \pm 0.8\%$ , and  $65.3 \pm 1.5\%$ , respectively. Even at the lowest dose of 0.04 mg/kg, ( $\pm$ )-**1**, (+)-**1**, (–)-**1**, and ( $\pm$ )-**3** still exhibited potent analgesic activities, comparable to morphine, with percentage inhibitions of  $37.2 \pm 0.8\%$ ,  $38.4 \pm 2.6\%$ ,  $30.8 \pm 2.3\%$ , and  $49.7 \pm 0.8\%$ , respectively.

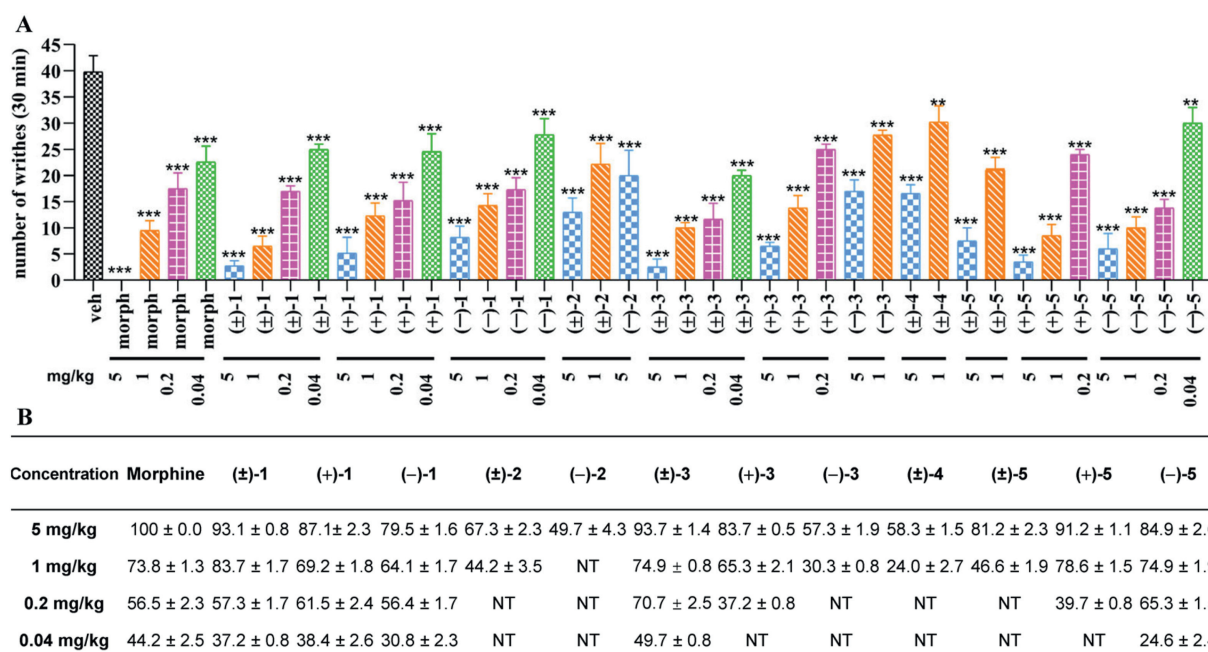
At doses of 5 and 1 mg/kg, ( $\pm$ )-**1** with a *cis*-orientation expressed higher analgesic percentage inhibition than its 4a,7a-epimer ( $\pm$ )-**2** with a *trans*-orientation. Thus, the *cis*-orientation may increase the analgesic activity.

Notably, (+)-**1** showed more potent analgesic activity than its enantiomer (–)-**1** at the tested four doses (Fig. 3). A similar trend was observed with (+)-**3** and (+)-**5**, both of which exhibited enhanced antinociceptive effects relative to their respective enantiomers (–)-**3** and (–)-**5**. Collectively, these findings underscore the critical role of chirality in dictating the analgesic potency.

Interestingly, the racemic mixture ( $\pm$ )-**1** exhibited superior analgesic activity compared to its pure enantiomers (+)-**1** and (–)-**1** at high doses of 5 and 1 mg/kg. At a dose of 5 mg/kg, ( $\pm$ )-**1** achieved a percentage inhibition of  $93.1 \pm 0.8\%$ , higher than those of (+)-**1** ( $87.1 \pm 2.3\%$ ) and (–)-**1** ( $79.5 \pm 1.6\%$ ). A similar trend was observed at a lower dose of 1 mg/kg. Thus, these two enantiomers (+)-**1** and (–)-**1** have a synergistic analgesic effect. The synergistic interaction observed in **1** was also found in **3** (Fig. 3), suggesting their structural features may favor cooperative binding or multi-target engagement. Therefore, the clinical use of racemic analgesics is more effective than any pure enantiomer, further proving the analgesic value of folk applications of the root extract of *A. pyrethrum*.

### 2.4. Analgesic targets investigation of (+)-1 and (–)-1

Many types of ion channels or receptors are involved in the regulation of pain. Among them, voltage-gated Kv1.2, Kv1.3, and Ca<sub>v</sub>2.1 ion channels, transient receptor potential melastatin 8



**Figure 3** Analgesic activities of **1–5** at a dose of 5.0, 1.0, 0.2, and 0.04 mg/kg with morphine (morph, at doses of 5.0, 1.0, 0.2, and 0.04 mg/kg) as a positive control. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  compared with the vehicle (veh), and data are expressed as mean  $\pm$  SEM of replicates. (A) Numbers of writhes in 30 min,  $n = 10$ . (B) Analgesic percentage inhibition, NT: not test.

(TRPM8) and canonical 6 (TRPC6), and the purinergic P2X7 receptor (P2X7R) have been identified as potential therapeutic targets for neuropathic pain. TRPM8 is a human cold-temperature thermosensor that is extensively expressed in the trigeminal ganglia and dorsal root ganglia and is involved in sensitized pain responses<sup>41</sup>. Kv1.2 and Kv1.3 regulate cold sensory thresholds by interacting with TRPM8 and play an important role in action potential conduction triggered by cold stimuli<sup>42,43</sup>. Cav2.1 plays key roles in regulating neuronal excitability, neurotransmitter release, and synaptic plasticity and is involved in the pathogenesis of epilepsy and neuropathic pain<sup>19,44</sup>. TRPC6 is a mechanosensitive channel expressed in dorsal root ganglia and glial cells and is involved in traumatic pain signaling<sup>14,15</sup>. P2X7R, which is widely expressed in microglia and neurons of the central nervous system, plays a critical role in the activation of signaling pathways, including PI3K/AKT, AMPK-PRAS40-mTOR, and the NLRP3 inflammasome signaling, making it a promising therapeutic target for inflammation and neuropathic pain<sup>45–48</sup>.

To explore their analgesic targets, the agonistic or inhibitory effects of (+)-**1** and (–)-**1** against a total of 44 analgesic-related ion channels and receptors including TRP, sodium, potassium, and calcium ion channels, as well as opioid, *N*-methyl-D-aspartate (NMDA), purinergic, and N-type cholinergic receptors were assessed through a whole-cell patch-clamp recording experiment (Fig. 4A and Supporting Information Tables S8–S19). The results demonstrated that (±)-**1**, (+)-**1** and (–)-**1** showed no significant activity at the  $\mu$ ,  $\kappa$ , and  $\delta$  opioid receptors (Fig. 4A and Tables S10–S12), suggesting their non-opioid analgesic potential.

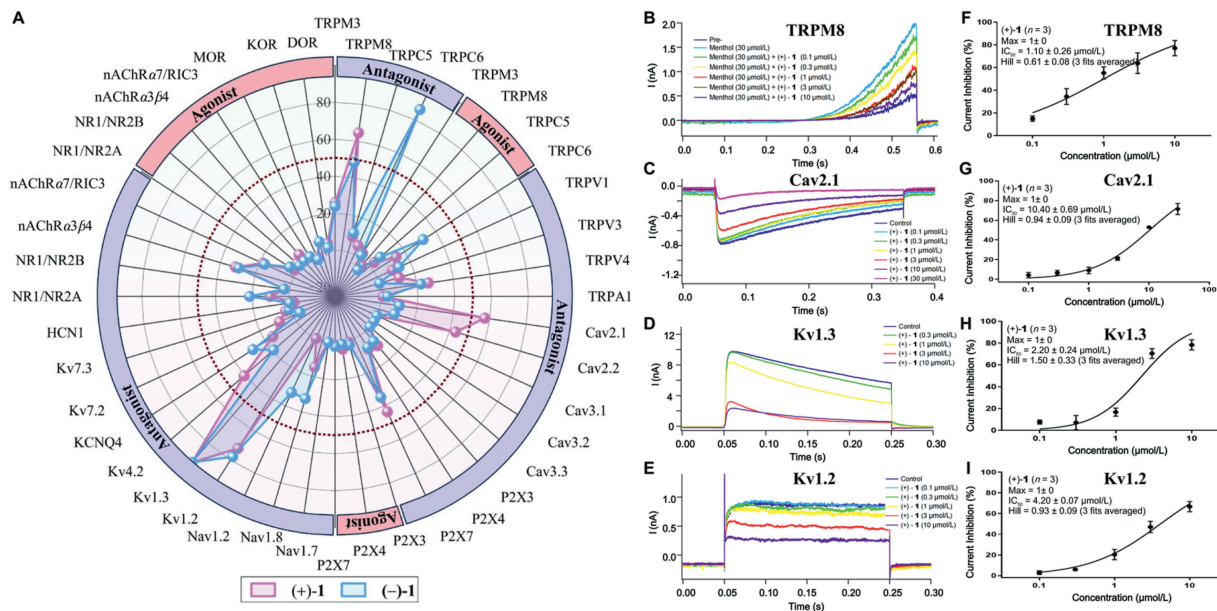
At a concentration of 10  $\mu$ mol/L, (+)-**1** significantly inhibited the peak currents of TRPM8, Cav2.1, Kv1.3, and Kv1.2, expressed in HEK 293T cells with percentage inhibitions of  $64.49 \pm 0.07\%$ ,  $57.26 \pm 0.08\%$ ,  $93.75 \pm 0.00\%$ , and  $72.55 \pm 0.03\%$ , respectively (Fig. 4A and Table S8), and their  $IC_{50}$  values were calculated to be

$1.10 \pm 0.26$ ,  $10.40 \pm 0.69$ ,  $4.20 \pm 0.07$ , and  $2.20 \pm 0.24$   $\mu$ mol/L (Fig. 4B–I and Supporting Information Figs. S9–S12, Table 1), respectively. In addition, (+)-**1** showed moderate inhibitory activities against TRPM3, Cav2.2, P2X7, Nav1.2, Kv4.2, and nAChR $\alpha 3\beta 4$  ion channels with percentage inhibitions ranging from 20% to 50%. Whereas, (–)-**1** showed significantly potent inhibitory effects against TRPC6, Kv1.2, and Kv1.3 ion channels with  $IC_{50}$  values of  $0.81 \pm 0.05$ ,  $0.91 \pm 0.04$ , and  $1.50 \pm 0.13$   $\mu$ mol/L (Table 1, Fig. S10, Fig. S11, and Supporting Information Fig. S13), respectively, and moderate inhibitory effects against TRPM3, TRPM8, P2X7, Nav1.8, NR1/NR2A, and nAChR $\alpha 3\beta 4$  with percentage inhibitions ranging from 20% to 50% (Fig. 4A–Tables S8 and S9). Thus, (+)-**1** and (–)-**1** exhibited potent analgesic properties by modulating multiple ion channels in the central nervous system.

Notably, at a concentration of 10  $\mu$ mol/L (Table 1 and Table S8), (+)-**1** exhibited more potent inhibitory effects on TRPM8, Cav2.1, and Cav2.2 with percentage inhibitions of  $57.26 \pm 0.08\%$ ,  $64.49 \pm 0.07\%$ , and  $43.27 \pm 0.07\%$ , respectively, than (–)-**1** with percentage inhibitions of  $48.36 \pm 0.06\%$ ,  $10.37 \pm 0.11\%$ , and  $8.63 \pm 0.01\%$ , respectively. This observation may explain the superior *in vivo* analgesic activity of (+)-**1** compared to (–)-**1**, although both of them expressed almost equivalent inhibitory activity on Kv1.2 and Kv1.3 ion channels.

Interestingly, the analgesic targets and inhibitory activity of these two enantiomers (+)-**1** and (–)-**1** are slightly different, and thus, their racemic form (±)-**1** would cause a synergistic analgesic effect. This could explain why the racemic mixture (±)-**1** showed more potent analgesic activity than either of the pure enantiomers (+)-**1** and (–)-**1**.

In summary, (+)-**1** and (–)-**1** demonstrated potent analgesic activity through synergistic modulation of multiple non-opioid pathways, including TRPM8, TRPC6, Kv1.2, Kv1.3, and Cav2.1 ion channels. Notably, (+)-**1** and (–)-**1** represent a structurally



**Figure 4** Inhibitory or agonistic effects of (+)-1 and (-)-1 on 44 analgesic-related targets, including TRP, sodium, potassium, and calcium ion channels, as well as opioid, *N*-methyl-D-aspartate, purinergic, and *N*-type cholinergic receptors, at the concentration of 10  $\mu\text{mol/L}$ ,  $n = 3$  (A); Inhibition of TRPM8 (B), Kv1.2 (C), Kv1.3 (D), and Cav2.1 (E) peak currents suppressed by different concentrations of (+)-1; Dose-response relationship and  $\text{IC}_{50}$  data of TRPM8 (F), Kv1.2 (G), Kv1.3 (H), and Cav2.1 (I) peak current inhibition of (+)-1.

**Table 1** Inhibitory effects of (+)-1 and (-)-1 on TRPM8, Kv1.2, Kv1.3, Cav2.1, and TRPC6 channels.

| Compounds                      | TRPM8                             | Kv1.2                               | Kv1.3                             | Cav2.1                             | TRPC6                             |
|--------------------------------|-----------------------------------|-------------------------------------|-----------------------------------|------------------------------------|-----------------------------------|
| (+)-1                          | $1.10 \pm 0.26 \mu\text{mol/L}^a$ | $4.20 \pm 0.07 \mu\text{mol/L}^a$   | $2.20 \pm 0.24 \mu\text{mol/L}^a$ | $10.40 \pm 0.69 \mu\text{mol/L}^a$ | $5.21\% \pm 0.02\%^b$             |
| (-)-1                          | $48.36\% \pm 0.06\%^b$            | $0.91 \pm 0.04 \mu\text{mol/L}^a$   | $1.50 \pm 0.13 \mu\text{mol/L}^a$ | $10.37\% \pm 0.11\%^b$             | $0.81 \pm 0.05 \mu\text{mol/L}^a$ |
| 2-APB <sup>c</sup>             | $3.90 \pm 0.36 \mu\text{mol/L}^a$ | NT                                  | NT                                | NT                                 | NT                                |
| 4-AP <sup>c</sup>              | NT                                | $111.70 \pm 11.0 \mu\text{mol/L}^a$ | NT                                | NT                                 | NT                                |
| Psora-4 <sup>c</sup>           | NT                                | NT                                  | $2.70 \pm 0.20 \text{ nmol/L}^a$  | NT                                 | NT                                |
| CdCl <sub>2</sub> <sup>c</sup> | NT                                | NT                                  | NT                                | $4.20 \pm 0.43 \mu\text{mol/L}^a$  | NT                                |
| SAR7334 <sup>c</sup>           | NT                                | NT                                  | NT                                | NT                                 | $5.90 \pm 0.89 \mu\text{mol/L}^a$ |

<sup>a</sup> $\text{IC}_{50}$  values are expressed as mean  $\pm$  SEM of triplicates.

<sup>b</sup>Percentage inhibition at a concentration of 10  $\mu\text{mol/L}$ .

<sup>c</sup>Positive control. NT: not test.

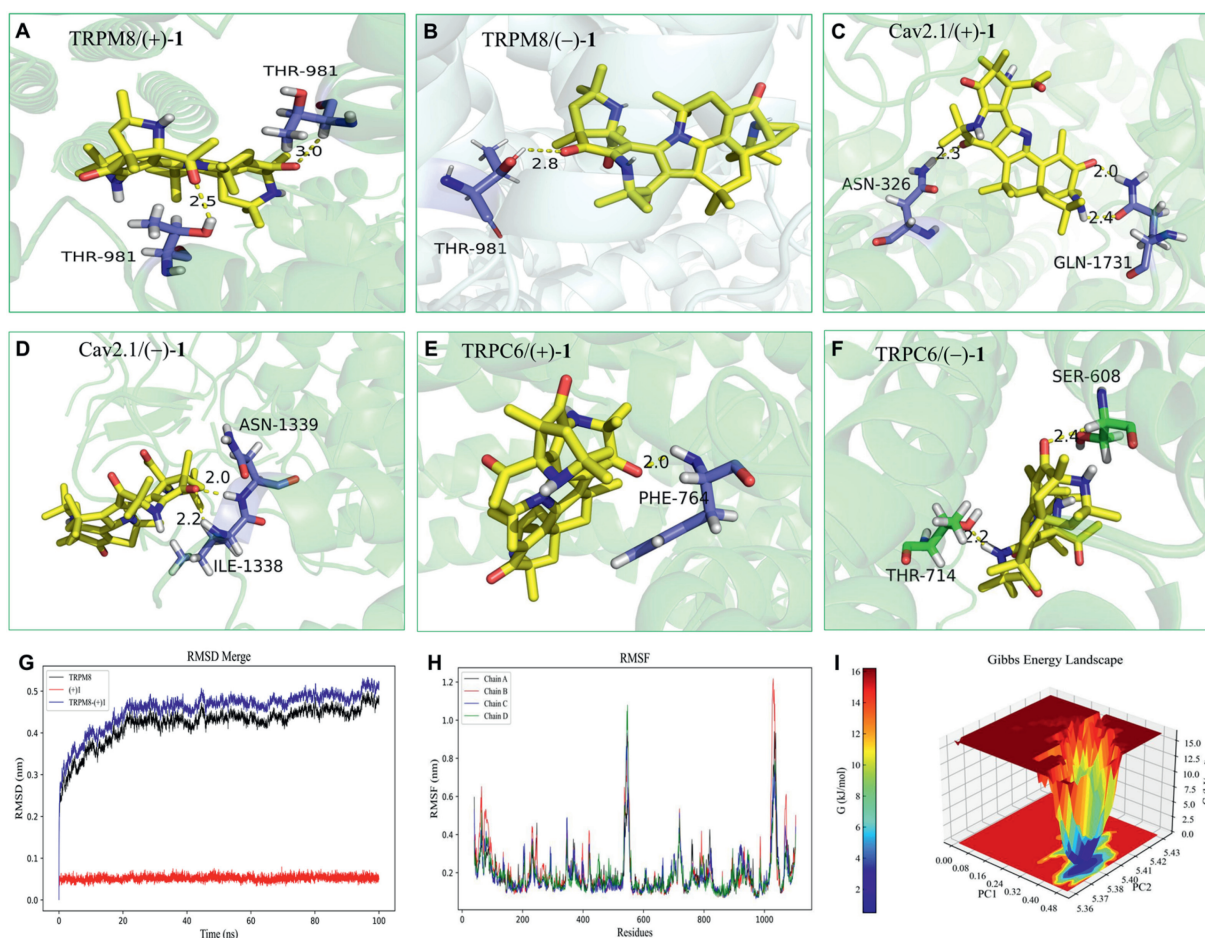
and mechanistically distinct class of multi-target non-opioid analgesics, contrasting fundamentally with recently developed opioid-based multi-target analgesics such as dezocine<sup>25</sup>, cebra-nopadol<sup>26</sup>, and BU08028<sup>27</sup>. The most efficacious racemic mixture, ( $\pm$ )-1, shows promising potential for development as a novel non-opioid analgesic to manage neuropathic, traumatic, and inflammatory pain syndromes. This pharmacological profile aligns with the traditional use of *A. pyrethrum* roots in folk medicine for treating cephalalgia, migraines, and cold-induced toothaches.

2.5. Docking analysis of (+)-1 and (-)-1 with TRPM8, TRPC6, and Cav2.1

To further explain the above inhibitory activity differences, the binding modes of (+)-1 and (-)-1 with TRPM8, TRPC6, and Cav2.1 were investigated by molecular docking<sup>32</sup> and molecular dynamics (MD) simulations<sup>49</sup> (Fig. 5, Supporting Information Figs. S14–S20 and Supporting Information Table S20).

The docking results (Fig. S14) demonstrated that TRPM8 exhibited distinct binding poses for (+)-1 and (-)-1. (+)-1 binds well to a more hydrophobic region of TRPM8, forming hydrophobic interactions with Try980 and Phe978 residues (Fig. S15). Based on this coordination, 18-carbonyl group in (+)-1 forms a hydrogen bond with the Thr981 residue, whereas this interaction was not observed in the binding mode of (-)-1 (Fig. S15). A computational analysis of the binding pocket of (+)-1 with TRPM8 indicates that the introduction of an appropriate volume of heteroatoms and hydrophobic groups at the 1- and 11-carbonyls positions and 7-NH may enhance its interaction with the protein, thereby improving the analgesic activity.

Regarding the binding modes of (+)-1 and (-)-1 to TRPC6 and Cav2.1, the molecular docking results demonstrated notable discrepancies in their binding sites (Fig. S14). This phenomenon may explain the apparent chiral specificity exhibited by (+)-1 and (-)-1 for TRPC6 and Cav2.1. Additionally, (+)-1 forms three hydrogen bonds with Cav2.1, one more than (-)-1, and its binding energy to Cav2.1 ( $-10.1 \text{ kcal/mol}$ ) is significantly lower than that



**Figure 5** Molecular docking and molecular dynamics simulation. Three-dimensional molecular docking models illustrating ligand interactions for TRPM8 binding to (+)-1 (A) and (-)-1 (B), Cav2.1 binding to (+)-1 (C) and (-)-1 (D), and TRPC6 binding to (+)-1 (E) and (-)-1 (F); (G) The RMSD curve of TRPM8/(+)-1 complex during the 100 ns all-atom MD simulation; (H) The protein backbone RMSF of TRPM8/(+)-1 complex during the 100 ns all-atom MD simulation; (I) Gibbs free energy 3D diagram of TRPM8/(+)-1 complex during the 100 ns all-atom MD simulation.

of (-)-1 (-8.1 kcal/mol) (Fig. S16 and Table S20). Similarly, (-)-1 forms two more hydrogen bonds with TRPC6 than (+)-1, and its binding energy to TRPC6 is significantly lower (-11.1 kcal/mol) than that of (+)-1 (-8.6 kcal/mol) (Fig. S17 and Table S20). These findings are consistent with the results of previous electrophysiology experiments.

To further confirm the binding mechanism, the atomistic molecular dynamic simulations of the complexes of TRPM8 and (+)-1 were performed. The results (Supporting Information Fig. S21) shown that the conformations of the complexes of (+)-1 and TRPM8 remained stable and did not significantly change. The high stability of the TRPM8 and (+)-1 complex was demonstrated by the root-mean-square deviation (RMSD) of <0.05 nm and the max root-mean-square fluctuation (RMSF) of <1.2 nm in the flexible regions.

### 3. Conclusions

In conclusion, five pairs of potent analgesic alkaloid enantiomers were discovered from the roots of *A. pyrethrum*. Among them, anacypyarethines A (1) and B (2) were identified to be highly conjugated and polymethylated 6/6/6/5/7/5/5-fused octacyclic

tetraazabic alkaloids, featuring an unprecedented 8,14,18,24-tetraaza-octacyclo[16.8.2.1<sup>1,23</sup>.0<sup>4,28</sup>.0<sup>5,17</sup>.0<sup>9,16</sup>.0<sup>11,15</sup>.0<sup>21,27</sup>]nonacosane motif. All the isolates demonstrated potent analgesic activity *in vivo*. (±)-1, (+)-5, and (-)-5 at a dose of 1.0 mg/kg and (±)-1, (+)-1, (±)-3, and (-)-5 at a dose of 0.2 mg/kg exhibited more potent analgesic activities than morphine. Interestingly, the racemic mixture (±)-1 showed stronger analgesic activity than either pure enantiomer alone at high doses of 5 and 1 mg/kg, and (+)-1 had stronger analgesic effect than (-)-1. A preliminary investigation of the mechanism of analgesic action demonstrated that (+)-1 mainly targeted TRPM8, Kv1.2, Kv1.3, and Cav2.1, while, (-)-1 targeted TRPC6, Kv1.2, and Kv1.3 ion channels, rather than the opioid receptors of morphine, suggesting their potential non-opioid analgesic properties and synergistic effects in analgesia. The molecular dockings of (+)-1 and (-)-1 offer a novel structural foundation for developing potent analgesics targeting TRPM8, TRPC6, Kv1.2, Kv1.3, and Cav2.1 ion channels. (±)-1 has the potential to be developed into a non-opioid potent analgesic to treat neuropathic, traumatic, and inflammatory pain conditions. The unprecedented chemical architecture, potent analgesic activity, and unique multiple analgesic targets of (+)-1 and (-)-1 will be promising candidates for interdisciplinary investigation across

the fields of synthetic chemistry, molecular pharmacology, and neurological research.

## 4. Experimental

### 4.1. General experimental procedures

Melting points were recorded on a BUCHI Melting Point B-540 (uncorrected). Optical rotations of the racemates and the enantiomers were tested on a Rudolph Research Analytical Autopol VI polarimeter with MeOH as solvent. IR spectra were determined in KBr disks on a Thermo NICOLET 6700 infrared spectrophotometer, and UV (MeOH as solvent) data were obtained by a Shimadzu UV-2550 spectrophotometer. ECD spectra of the enantiomers in MeOH were recorded on an Applied Photophysics Chirascan spectropolarimeter. NMR spectra were recorded on Bruker AM-400 and AM-600 spectrometers, and chemical shift values were provided in  $\delta$  (ppm) referred to the residual signals of chloroform-*d* ( $\delta_{\text{H}}$  7.24 and  $\delta_{\text{C}}$  77.23), pyridine-*d*<sub>5</sub> ( $\delta_{\text{H}}$  8.74, 7.58, 7.22, and  $\delta_{\text{C}}$  150.35, 135.91, 123.87), acetone-*d*<sub>6</sub> ( $\delta_{\text{H}}$  2.05, and  $\delta_{\text{C}}$  206.68, 29.92), DMSO-*d* ( $\delta_{\text{H}}$  2.50, and  $\delta_{\text{C}}$  39.51). <sup>15</sup>N NMR spectra measured at 40 MHz in pyridine-*d*<sub>5</sub> on Bruker AM-400, and chemical shift values were provided in  $\delta$  (ppm) referred to the residual signals of pyridine-*d*<sub>5</sub> ( $\delta_{\text{N}}$  319). X-ray crystallographic data were obtained on a Rigaku XtaLAB PRO MM007HF or Rigaku Oxford Diffraction Ltd. or Bruker D8 VENTURE single-crystal X-ray diffractometer equipped with graphite-monochromatized Cu K $\alpha$  radiation ( $\lambda$  = 1.54184 Å). HRESIMS data were measured by a QSTAR Elite LC–MS/MS spectrometer. Semipreparative HPLC separations were carried out on a Hanbon NP7005C equipped with a NU3001D detector, and Shimadzu LC-20 AT equipped with an SPD-20AT PDA detector. A DAICEL CORPORATION Chiralpak ID column (5  $\mu$ m, 10 mm  $\times$  50 mm, i.d.) was used for HPLC purification. Silica gel (100–200 and 300–400 mesh, Yantai JiangYou Silica Gel Development Co., Ltd., Yantai, China), MCI gel (CHP20P, 75–150  $\mu$ m, Mitsubishi Chemical Industries Ltd., Tokyo, Japan), reversed-phase C18 (Rp-C18) silica gel (12 nm, S-50  $\mu$ m, YMC Co., Ltd.), and Sephadex LH-20 gel (Pharmacia Uppsala, Sweden) were used for column chromatography (CC). Solvents used in CC and HPLC were of analytical grade (Tianjin Xibote Chemical Co. Ltd., Tianjin, China). Solvents used in HPLC were of LC–MS grade (Merck, Germany).

### 4.2. Plant material

The root of *A. pyrethrum* (L.) DC was purchased from the Xinjiang Ensar Uygur Pharmaceutical Co., Ltd. (People's Republic of China) in October 2020, and was authenticated by Associate Professor Chunfang Lu at Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences (CAS). A voucher specimen (number: WY02684) has been deposited at the Specimen Museum of Xinjiang Institute of Ecology and Geography, CAS.

### 4.3. Extraction and isolation

The roots of *A. pyrethrum* (15.0 kg) were powdered and extracted with MeOH (25 L  $\times$  8, 24 h for each time) at room temperature (r.t). The crude extract (2.2 kg) was suspended in 5% HCl (2 L) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (10 L  $\times$  3) to remove the nonalkaloid

components. The aqueous layer was basified with saturated NaHCO<sub>3</sub> solution in an ice water bath to pH 10 and extracted with CH<sub>2</sub>Cl<sub>2</sub> to give 700.0 g total alkaloids.

The detailed isolation of **1–5** was described in the Supporting Information

### 4.4. Physical constants, spectrometric, and spectroscopic data

(±)-Anacyphrethine A (**1**): yellow acicular crystals, m.p. 244–247 °C;  $[\alpha]_{\text{D}}^{25} +1$  (*c* 0.1, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 427 (4.15) and 267 (3.96) nm; IR (KBr) max 3297, 2961, 2924, 1704, 1623, and 1448 cm<sup>−1</sup>; HRESIMS *m/z* 641.4051 [*M*  $\pm$  H]<sup>+</sup> (Calcd. for C<sub>39</sub>H<sub>53</sub>O<sub>4</sub>N<sub>4</sub><sup>+</sup>, 641.4066); <sup>1</sup>H and <sup>13</sup>C NMR data, Table S1. The relevant spectra are presented in Supporting Information Figs. S21–S53.

(+)-Anacyphrethine A (**1**): yellow acicular crystals, m.p. 223–224 °C;  $[\alpha]_{\text{D}}^{25} +1584$  (*c* 0.08, MeOH) (Supporting Information Fig. S54); ECD (*c* 3.12  $\times$  10<sup>−4</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 216 (−11.39), 253 (−2.36), 289 (−13.16), and 417 (+17.07) nm.

(−)-Anacyphrethine A (**1**): yellow acicular crystals, m.p. 222–223 °C;  $[\alpha]_{\text{D}}^{25} -1584$  (*c* 0.1, MeOH) (Supporting Information Fig. S55); ECD (*c* 3.12  $\times$  10<sup>−4</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 217 (+9.25), 253 (+0.05), 290 (+13.67), and 416 (−21.25) nm.

(±)-Anacyphrethine B (**2**): yellow acicular crystals, m.p. 219–220 °C;  $[\alpha]_{\text{D}}^{25} -1$  (*c* 0.1, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 475 (3.90) and 281 (3.74) nm; IR (KBr) max 3316, 2963, 2926, 2857, 1699, 1615, 1445, and 1194 cm<sup>−1</sup>; HRESIMS *m/z* 641.4049 [*M*  $\pm$  H]<sup>+</sup> (Calcd. for C<sub>39</sub>H<sub>53</sub>O<sub>4</sub>N<sub>4</sub><sup>+</sup>, 641.4066); <sup>1</sup>H and <sup>13</sup>C NMR data, Table S3. The relevant spectra are presented in Supporting Information Figs. S56–S83.

(+)-Anacyphrethine B (**2**): yellow amorphous powder,  $[\alpha]_{\text{D}}^{25} +550$  (*c* 0.02, MeOH) (Supporting Information Fig. S84); ECD (*c* 3.12  $\times$  10<sup>−4</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 220 (30.48), 264 (−25.61), 301 (+46.37), 380 (−34.83), and 480 (+10.83) nm.

(−)-Anacyphrethine B (**2**): yellow amorphous powder,  $[\alpha]_{\text{D}}^{25} -550$  (*c* 0.03, MeOH) (Supporting Information Fig. S85); ECD (*c* 3.12  $\times$  10<sup>−4</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 219 (−23.50), 265 (+17.79), 300 (−35.20), 381 (+25.45), and 481 (−9.33) nm.

(±)-Anacyphrethine C (**3**): colorless block crystals, m.p. 172–173 °C;  $[\alpha]_{\text{D}}^{25} +2$  (*c* 0.1, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 328 (0.88) nm; HRESIMS *m/z* 305.2221 [*M*  $\pm$  H]<sup>+</sup> (Calcd. for C<sub>18</sub>H<sub>29</sub>O<sub>2</sub>N<sub>2</sub>, 305.2224); <sup>1</sup>H and <sup>13</sup>C NMR data, Table S5. The relevant spectra are presented in Supporting Information Figs. S86–S94.

(+)-Anacyphrethine C (**3**): colorless oil,  $[\alpha]_{\text{D}}^{25} +82$  (*c* 0.1, MeOH) (Supporting Information Fig. S95); ECD (*c* 3.15  $\times$  10<sup>−3</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 205 (+8.75), 234 (+0.72), and 263 (+5.02) nm.

(−)-Anacyphrethine C (**3**): colorless oil,  $[\alpha]_{\text{D}}^{25} -82$  (*c* 0.1, MeOH) (Supporting Information Fig. S96); ECD (*c* 3.15  $\times$  10<sup>−3</sup>, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ) 203 (−8.69), 234 (−0.56), and 263 (−4.81) nm.

(±)-Pyracyclumine B (**4**): yellow amorphous powder;  $[\alpha]_{\text{D}}^{25} +1$  (*c* 0.1, MeOH); HRESIMS *m/z* 287.2115 [*M*  $\pm$  H]<sup>+</sup> (Calcd. for C<sub>18</sub>H<sub>27</sub>ON<sub>2</sub>, 287.2115); <sup>1</sup>H and <sup>13</sup>C NMR data, Table S6. The relevant spectra are presented in Supporting Information Figs. S97–S103.

(+)-Pyracyclumine B (**4**): yellow amorphous powder,  $[\alpha]_{\text{D}}^{25} +338$  (*c* 0.04, MeOH) (Supporting Information Fig. S104).

(−)-Pyracyclumine B (**4**): yellow amorphous powder,  $[\alpha]_{\text{D}}^{25} -338$  (*c* 0.04, MeOH) (Supporting Information Fig. S105).

(±)-Pyracyclumine A (**5**): colorless block crystals, m.p. 168–169 °C;  $[\alpha]_{\text{D}}^{25} -2$  (*c* 0.1, MeOH); HRESIMS *m/z* 343.2376 [*M*  $\pm$  H]<sup>+</sup> (Calcd. for C<sub>21</sub>H<sub>31</sub>O<sub>2</sub>N<sub>2</sub>, 343.2380); <sup>1</sup>H and <sup>13</sup>C NMR

data, Table S7. The relevant spectra are presented in Supporting Information Figs. S106–S112.

(+)-Pyracyclumine A (**5**): colorless oil,  $[\alpha]_D^{25} +69$  (*c* 0.1, MeOH) (Supporting Information Fig. S113).

(-)-Pyracyclumine A (**5**): colorless oil,  $[\alpha]_D^{25} -69$  (*c* 0.1, MeOH) (Supporting Information Fig. S114).

#### 4.5. Single crystal X-ray diffraction analysis and crystallographic data for compounds ( $\pm$ )-**1**, (+)-**1**, (-)-**1**, ( $\pm$ )-**2**, ( $\pm$ )-**3**, and ( $\pm$ )-**5**

X-ray crystallographic data for ( $\pm$ )-**1**, (+)-**1**, and (-)-**1** were obtained using a Rigaku XtaLAB PRO MM007HF and a Rigaku Oxford Diffraction Ltd. instrument with graphite-monochromatized Cu K $\alpha$  radiation ( $\lambda = 1.54184$  Å), and the X-ray crystallographic data for ( $\pm$ )-**2**, ( $\pm$ )-**3**, and ( $\pm$ )-**5** were obtained using the Rigaku Oxford Diffraction Ltd. instrument. The crystal structures of ( $\pm$ )-**1**, (+)-**1**, (-)-**1**, ( $\pm$ )-**2**, ( $\pm$ )-**3** and ( $\pm$ )-**5** were solved and refined using the Olex2 software with the ShelXT and ShelXL programs, respectively. All non-hydrogen atoms were refined anisotropically using the full-matrix least-squares method. The Ortep drawing with 10% ellipsoid probability level of the crystal structures of ( $\pm$ )-**1**, (+)-**1**, (-)-**1**, ( $\pm$ )-**2**, ( $\pm$ )-**3**, and ( $\pm$ )-**5** were depicted in Fig. 2.

Crystallographic data for (+)-**1**: yellow acicular crystals of (+)-**1** were recrystallized in CH<sub>3</sub>CN/H<sub>2</sub>O (10 : 1), C<sub>39</sub>H<sub>52</sub>O<sub>4</sub>N<sub>4</sub> (*M* = 640.41 g/mol): space group *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, *a* = 8.0833 (5) Å, *b* = 24.4709 (16) Å, *c* = 35.3330 (2) Å,  $\alpha = 90^\circ$ ,  $\beta = 90^\circ$ ,  $\gamma = 90^\circ$ , *V* = 4713.42(9) Å<sup>3</sup>, *Z* = 4, *T* = 99.99 (10) K,  $\mu$ (Cu K $\alpha$ ) = 0.624 mm<sup>-1</sup>,  $\rho_{\text{calc}} = 1.218$  mg/mm<sup>3</sup>, 71,925 reflections measured ( $4.392^\circ \leq 2\theta \leq 154.4^\circ$ ), 14,524 independent reflections ( $R_{\text{int}} = 0.0588$ ) which were used in all calculations. The final *R*<sub>1</sub> was 0.0369 (*I*  $\geq 2\sigma$  (*I*)) and *wR*<sub>2</sub> was 0.0960 (*I*  $\geq 2\sigma$  (*I*)). The final *R*<sub>1</sub> was 0.0383 (all data) and *wR*<sub>2</sub> was 0.0970 (all data). The goodness of fit on *F*<sup>2</sup> was 1.046. The Flack parameter was -0.01(5).

Crystallographic data for (-)-**1**: yellow acicular crystals of (-)-**1** were recrystallized in CH<sub>3</sub>CN/H<sub>2</sub>O (10:1), C<sub>39</sub>H<sub>52</sub>O<sub>4</sub>N<sub>4</sub> (*M* = 640.84 g/mol): space group *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, *a* = 8.08222 (4) Å, *b* = 24.47359 (12) Å, *c* = 35.32707 (2) Å,  $\alpha = 90^\circ$ ,  $\beta = 90^\circ$ ,  $\gamma = 90^\circ$ , *V* = 6987.73(6) Å<sup>3</sup>, *Z* = 8, *T* = 100.00 (10) K,  $\mu$ (Cu K $\alpha$ ) = 0.624 mm<sup>-1</sup>,  $\rho_{\text{calc}} = 1.218$  g/cm<sup>3</sup>, 78,368 reflections measured ( $5.002^\circ \leq 2\theta \leq 148.682^\circ$ ), 14,039 independent reflections ( $R_{\text{int}} = 0.0360$ ,  $R_{\text{sigma}} = 0.0234$ ) which were used in all calculations. The final *R*<sub>1</sub> was 0.0287 (*I*  $\geq 2\sigma$  (*I*)) and *wR*<sub>2</sub> was 0.0736 (*I*  $\geq 2\sigma$  (*I*)). The final *R*<sub>1</sub> was 0.0295 (all data) and *wR*<sub>2</sub> was 0.0741 (all data). The goodness of fit on *F*<sup>2</sup> was 1.012. The Flack parameter was 0.01(4).

Crystallographic data for ( $\pm$ )-**2**: yellow acicular crystals of **2** were recrystallized in CH<sub>3</sub>CN/H<sub>2</sub>O (10 : 1), C<sub>39</sub>H<sub>53</sub>O<sub>4</sub>N<sub>4</sub> (*M* = 640.84 g/mol): space group *P*-1, *a* = 9.3614 (3) Å, *b* = 13.8454 (5) Å, *c* = 14.5058 (4) Å,  $\alpha = 98.9^\circ$ ,  $\beta = 108.2^\circ$ ,  $\gamma = 93.5^\circ$ , *V* = 1752.27(10) Å<sup>3</sup>, *Z* = 2, *T* = 170.0 K,  $\mu$ (Cu K $\alpha$ ) = 0.622 mm<sup>-1</sup>,  $\rho_{\text{calc}} = 1.215$  g/cm<sup>3</sup>, 16,059 reflections measured ( $6.506^\circ \leq 2\theta \leq 133.512^\circ$ ), 6128 independent reflections ( $R_{\text{int}} = 0.0642$ ,  $R_{\text{sigma}} = 0.0720$ ) which were used in all calculations. The final *R*<sub>1</sub> was 0.0623 (*I*  $\geq 2\sigma$  (*I*)) and *wR*<sub>2</sub> was 0.1342 (*I*  $\geq 2\sigma$  (*I*)). The final *R*<sub>1</sub> was 0.1044 (all data) and *wR*<sub>2</sub> was 0.1630 (all data). The goodness of fit on *F*<sup>2</sup> was 1.042.

Crystallographic data for ( $\pm$ )-**3**: colorless block crystals of **3** were recrystallized in acetone, C<sub>18</sub>H<sub>28</sub>O<sub>2</sub>N<sub>2</sub> (*M* = 304.42 g/mol):

space group *P*2<sub>1</sub>/c, *a* = 7.1973(3) Å, *b* = 10.8034(5) Å, *c* = 21.9721(12) Å,  $\alpha = 90^\circ$ ,  $\beta = 91.174(3)^\circ$ ,  $\gamma = 90^\circ$ , *V* = 1708.09(14) Å<sup>3</sup>, *Z* = 4, *T* = 170.0 K,  $\mu$ (Cu K $\alpha$ ) = 0.609 mm<sup>-1</sup>,  $\rho_{\text{calc}} = 1.184$  g/cm<sup>3</sup>, 11,348 reflections measured ( $8.05^\circ \leq 2\theta \leq 149.168^\circ$ ), 3393 independent reflections ( $R_{\text{int}} = 0.0569$ ,  $R_{\text{sigma}} = 0.0499$ ) which were used in all calculations. The final *R*<sub>1</sub> was 0.0474 (*I*  $\geq 2\sigma$  (*I*)) and *wR*<sub>2</sub> was 0.1117 (*I*  $\geq 2\sigma$  (*I*)). The final *R*<sub>1</sub> was 0.0652 (all data) and *wR*<sub>2</sub> was 0.1251 (all data). The goodness of fit on *F*<sup>2</sup> was 1.042.

Crystallographic data for ( $\pm$ )-**5**: colorless block crystals of **5** were recrystallized in methanol, C<sub>21</sub>H<sub>30</sub>O<sub>2</sub>N<sub>2</sub> (*M* = 342.48 g/mol): space group *P*2<sub>1</sub>/c, *a* = 13.2553(4) Å, *b* = 20.9558(7) Å, *c* = 15.1314(4) Å,  $\alpha = 90^\circ$ ,  $\beta = 113.9070(10)^\circ$ ,  $\gamma = 90^\circ$ , *V* = 3842.5(2) Å<sup>3</sup>, *Z* = 4, *T* = 100(2) K,  $\mu$ (Cu K $\alpha$ ) = 0.628 mm<sup>-1</sup>,  $\rho_{\text{calc}} = 1.215$  g/cm<sup>3</sup>, 37,133 reflections measured ( $7.502^\circ \leq 2\theta \leq 149.094^\circ$ ), 7805 independent reflections ( $R_{\text{int}} = 0.0770$ ,  $R_{\text{sigma}} = 0.0529$ ) which were used in all calculations. The final *R*<sub>1</sub> was 0.0498 (*I*  $\geq 2\sigma$  (*I*)) and *wR*<sub>2</sub> was 0.1290 (*I*  $\geq 2\sigma$  (*I*)). The final *R*<sub>1</sub> was 0.0582 (all data) and *wR*<sub>2</sub> was 0.1375 (all data). The goodness of fit on *F*<sup>2</sup> was 1.013.

The crystallographic data have been deposited to the Cambridge Crystallographic Data Centre with CCDC numbers: 2359861–2359864 and 2359866–2359867, respectively. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223–336033 or email: deposit@ccdc.cam.ac.uk).

#### 4.6. LC–MS/MS and molecular networking analysis

HPLC analyses were performed with a Dionex UltiMate 3000 RSLCnano System equipped with an Xselect CSH C18 column (4.6 mm  $\times$  250 mm; 5  $\mu$ m, Waters). The mobile phase system was solvent A (0.1% ammonium formate/H<sub>2</sub>O) and solvent B (acetonitrile) using the following gradient: 0–140 min (10%–40% B), 140–240 min (40%–80% B), 240–260 min (80%–100% B) while stabilization time was 10 min, the flow rate was 1 mL/min, injection volume 20  $\mu$ L.

LC–ESI–HRMS analyses were achieved by coupling the LC system to a Thermo Scientific Q Exactive Plus Orbitrap equipped with an ESI dual source, operating in positive-ion mode. The following adjustments were made to the electrospray ionization (ESI) source parameters: high collision dissociation cell (HCD) energy, 27 eV (for CAM106) and 42 eV (for the IS); capillary temperature, 320  $^\circ$ C; S-lens RF level, 55 V; automatic gain control (AGC) target, 1e6; maximum IT, 100 ms; spray voltage, 3.2 kV. The HCD energy was normalized collision energy (NCE): 20, 40, and 60 eV. With a resolution of 17,500, the complete MS/dd MS2 mode was used to find the target alkaloids.

The molecular networks were created using the online workflow at Global Natural Products Social molecular networking (<http://gnps.ucsd.edu>)<sup>50</sup>. MS2 spectra were window-filtered by choosing only the top six peaks in the  $\pm 50$  Da window throughout the spectrum. The data were not clustered with MS-Cluster. A network was created where edges were filtered to have a cosine score above 0.7 and at least four matching peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 4 matched peaks.

#### 4.7. NMR calculation and DP4+ analysis of **1** and **2**

The NMR calculation and DP4+ analysis of **1** and **2** was conducted as previously described<sup>35,36</sup>. The BALLOON program was used to perform semi-empirical PM3 quantum mechanical geometry optimizations on the plausible stereoisomers **1a**, **1b**, **2a**, and **2b**. The stable conformers were then screened based on their energy values of the optimized structures calculated at the B3LYP/6-31G(d) level with the Gaussian 09 program. After optimization using the B3LYP/6-31G(d) method in the gas phase, all selected conformers for NMR calculations were computed *via* the Gauge-Independent Atomic Orbital (GIAO) method combined with the mPW1PW91/6-311G(d,p) basis set for **1a**, **1b**, **2a**, and **2b**, again using the Gaussian 09 program. Finally, the DP4+ probability method was applied to analyze the computed NMR data of the four isomers to determine the most plausible relative configurations of compounds **1** and **2**.

#### 4.8. Quantum chemical ECD calculations of **1–3**

ECD calculations for anacyphrethines A–C (**1–3**) were performed as previously described<sup>32,33,37,38</sup>. Conformational searching of compounds **1–3** were performed with the Crest code (version 2.11) using the default iMTD-GC procedure<sup>51</sup>. Those two conformers with difference in distance matrix below 0.5 Å were regarded as duplicate conformers, and the one with higher energy was removed. After clustering, those conformers within an energy cutoff of 5 kcal/mol were subjected to DFT geometry optimizations at M06-2X-D3/def2-SVP in the gas phase. Frequency analysis of all optimized conformers was undertaken at the same level of theory to ensure they were true local minima on the potential energy surface. Then, energies of all optimized conformers were evaluated at M06-2X-D3/def2-TZVP level of theory in the gas phase. Gibbs free energy of each conformer was calculated by adding thermal correction to Gibbs free energy obtained by frequency analysis to electronic energy obtained at M06-2X-D3/def2-TZVP level of theory. Room-temperature (298.15 K) equilibrium population of each conformer was calculated according to the Boltzmann distribution law. The geometry optimization, single-point energy calculations, ECD calculations were all completed in Gaussian 09 program. Detailed calculation data can be found in Supporting Information Table S21–S31.

#### 4.9. Analgesic activity evaluation of the isolates **1–5** in an acetic acid-induced writhing test in mice

The analgesic activities of the isolates **1–5** were evaluated using an acetic acid-induced writhing test in mice as previously described<sup>33,40</sup>. Mice were obtained from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology, 4-week-old Kunming mice of both sexes (20–30 g). The license number for the use of experimental animals is No. SCXK (Hubei) 2021–0057, and the license number of experimental animal production is No. SCXK (Hubei) 2020–0018.

The mice were randomly allocated to cages, weighed and labelled, with ten mice in each group ( $n = 10$ ). Prior to the commencement of the experiment, the mice were placed in the test environment for a period of 2 h to allow for acclimatisation. During the formal experiments, mice were administered intraperitoneally according to groups. The blank group was given a corresponding volume (10 mL/kg body weight) of 0.9% sodium

chloride solution. Different doses were set for each administration group by referring to the administration doses reported in the literature, combined with the mass obtained from the compounds themselves. After 30 min of intraperitoneal administration, the mice were intraperitoneally injected with the acetic acid solution at a concentration of 0.6% *v/v* (0.1 mL/10 g) (ready to use). The mice were then observed for the number of torsions over a 30-min period. A torsion was defined as a contraction of the abdominal muscles, rotation of the trunk (twisting), and extension of the body and hind limbs. Morphine was employed as a positive control drug.

Data were analyzed and plotted using GraphPad Prism 8 and variance and *P*-values were calculated (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). The percentage inhibition was calculated by subtracting the number of torsions in the administered group from the number of torsions in the blank group and dividing the result by the number of torsions in the blank group. This value is expressed as a percentage.

In general, the analgesic activities of the isolates were initially evaluated at a dose of 5 mg/kg. If the analgesic percentage inhibition exceeds 50%, the analgesic activity would be tested at lower doses of 1, 0.2, and 0.04 mg/kg, respectively.

#### 4.10. Bioassay of the isolates **1–5** against 44 analgesic-related receptors and ion channels

The details were described in the Supporting Information

#### 4.11. Molecular docking analysis

TRPM8 (PDB ID: 8E4Q)<sup>52</sup>, Kv1.2 (PDB ID: 5WIE)<sup>53</sup>, Kv1.3 (PDB ID: 7EJ1)<sup>54</sup>, Cav2.1 (PDB ID: 8X90)<sup>55</sup>, and TRPC6 (PDB ID: 7DXF)<sup>56</sup> were downloaded from the Protein Data Bank (<http://www.rcsb.org>). The protein preparation wizard in AutoDock was used to prepare them by taking off the water molecules and adding hydrogen atoms<sup>32</sup>. Compounds (+)-**1** and (–)-**1** were docked with TRPM8, Kv1.2, Kv1.3, Cav2.1, and TRPC6 following energy minimization. The binding site was determined to be a sphere with a radius of 10 Å surrounding the template molecule prior to docking. Every parameter was left at its default setting for the simulated annealing. The docking process produced 10 of the best ligand–receptor conformations, and AutoDock's receptor–ligand interactions were used to visualize and evaluate the docked molecules' binding patterns.

#### 4.12. Molecular dynamics simulation analysis

Molecular dynamics (MD) simulation of the TRPM8 (PDB ID: 8E4Q) bound to compound (+)-**1** in an aqueous solution was conducted using Gromacs 2022.3. Ligands were preprocessed with AmberTools22<sup>49</sup>. The system was maintained at a constant temperature of 300 K and pressure of 1 bar. The Amber99sb-ildn force field was employed, and the solvent environment was modeled with the TIP3P water model, with counterions ( $\text{Na}^+$ ) added to neutralize the system. The workflow included energy minimization *via* the steepest descent algorithm to eliminate conformational stresses, followed by NVT (constant particle number, volume, temperature) and NPT (constant particle number, pressure, temperature) equilibration (100 ps each), and a 100-ns production MD simulation (50 million steps, 2 fs timestep). Trajectory analysis incorporated root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), hydrogen

bond dynamics, Gibbs free energy landscapes, and MM/GBSA binding energy calculations to assess structural stability, flexibility, and binding thermodynamics. All parameters were set as default.

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## Author contributions

This work was designed and guided by Haji Akber Aisa and Guangmin Yao, the funding was obtained by Haji Akber Aisa and Guangmin Yao. The manuscript was written and revised by Hui Chen, Guangmin Yao, and Haji Akber Aisa. Hui Chen and Bianlin Wang conducted the separation, structural identification, and ion channel experiments. Hanqi Zhang, Biao Gao, and Zhijun Liu finished the acetic acid-induced writhing analgesia experiment in mice. Bao Gao and Zhijun Liu conducted the ECD calculations. Chao Niu conducted the molecular docking and molecular dynamics simulations calculations. Guangmin Yao proposed the biosynthetic pathway. All authors read and approved the final manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

## Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.04.032>.

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